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THERAPEUTIC USE OF CERTAIN AZO-DYES IN EXPERIMENTALLY PRODUCED TUBERCULOSIS IN GUINEA-PIGS.*

STUDIES ON THE BIOCHEMISTRY AND CHEMOTHERAPY OF TUBERCULOSIS. VIII.

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This report presents observations made during the last two years in a series of experiments, described in part in an earlier article[1] and continued and extended since the publication of that paper. As is well known, trypan red is the dye first reported by Ehrlich and Shiga in 1904 as having a "Magna sterilans" action in certain trypanosome infections.[2] Numerous investigators have since that time been working with this dye and its companion, trypan blue, and other related dyes, and an extensive literature has developed. Much of this literature, however, was reviewed briefly in my preliminary communication and it has recently been reviewed by Henius[3] and others, so that it seems unnecessary at this time to do more than mention the work which deals with the relation of these dyes to tuberculosis. Goldmann published several monographs on the distribution of the dyes and their use in the study of cells, and in 1912[4] he made an extensive study of bovine and avian tuberculosis in white mice, especially with respect to the development of the disease and the histology and histogenesis of the tubercle. Bowman, Winternitz, and Evans[5] in 1912 and in 1914 made observations with these dyes on the histogenesis of tubercles and tuberculous giant cells. Lewis[6] in

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[1] *Jour. Infect. Dis.*, 1913, 13, p. 378.

[2] *Berl. klin. Wchnschr.*, 1904, 41, pp. 329 and 362.

[3] *Ztschr. f. Chemoth.*, II, *Ref.* 1913, 2, 1037, and 1914, 2, 1097.

[4] *Beitr. z. klin. Chir.*, 1912, 78, p. 1.

[5] *Centralbl. f. Bakteriol.*, I, *Orig.*, 1912, 65, p. 403, and *Jour. Exp. Med.*, 1914, 19, p. 283.

[6] *Arch. Int. Med.*, 1912, 10, p. 68.

1912 was able to show that trypan red penetrated to the center of fully formed tubercles in rabbits. In 1912[1] also he presented before the American Society for Pharmacology and Experimental Therapeutics a brief report of his early work on modifications of the trypan red moleculi. Compounds of trypan red with iodin, thymus, eucalyptol, guaiacol, and iodoform were prepared and found to have similar powers of penetrating the tubercle. But neither trypan red nor these modifications had any appreciable influence in prolonging the course of the infection. In my earlier paper I showed that both trypan blue and trypan red penetrated tubercles in guinea-pigs, even the soft caseous substance in the centers of tuberculous lymph glands being stained deeply blue or red, and the large necrotic areas so common in the spleen and liver in these animals taking the stain especially well. The tubercles were found well stained a few hours after a single dye injection and retained the stain for many weeks after the last injection. The tubercle bacilli, however, were shown to be but poorly stained by these dyes in the animal body, in the culture tube, or when fixed to a slide; it was also found that the dyes had practically no bactericidal power over these organisms, since guinea-pigs inoculated with bacillary suspensions which had been exposed for 24 hrs. to 1 per cent or even 2 or 3 per cent solutions quickly developed the disease and died with a local and general tuberculosis. The dyes were found lacking even in inhibitory influence, since a luxuriant growth developed on agar tubes which were deeply dyed.

However, as Ehrlich and Shiga had shown that trypan red failed to kill trypanosomes *in vitro* and yet was a very efficient trypanocide *in vivo*, it was thought possible that a similar finding might be obtained in the treatment of tuberculosis. Hence numerous guinea-pigs which had been recently inoculated with human tuberculosis were treated with trypan blue or trypan red; 1 per cent water solutions of the dyes were injected subcutaneously, 3–6 c.c. constituting the dose according to the size of the animal. The injections were repeated once or twice a week and ill effects were rarely observed. Table 1 (p. 500) gives briefly the results.

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[1] *Jour. of Pharmacol. and Exper. Therap.*, 1912–13, 4, p. 353.

In all these guinea-pigs the dyes had penetrated the tubercles well. Even the softened caseous substance in the center of the tuberculous lymph glands was deeply stained, while the smaller tubercles had the characteristic structure. At the periphery were cells with coarse, deeply stained granules, differing from the normal granular cells in the size and irregularity of the granules.

TABLE 1.

SHOWING RESULT OF INJECTION OF 4 C.C. OF 1 PER CENT TRYPAN BLUE.

Time between Inoculation and First Dye Injection	Number of Injections	Time between Inoculation and Death	Autopsy Findings	Effect of Dye Treatment
Days		Days		
1	9	35 (killed)	Loc. and gen. tbc.	None
1	9	70	" "	"
1	2	10	No tbc.	Probably caused abortion
1	2	8	No tbc. Pneumonia	None
1	8	72	Loc. and gen. tbc.	"
62	2	72 (killed)	" "	"
62	2	72 "	" "	"
62	2	72 "	" "	"
62	5	104	" "	"
62	2	72 (killed)	" "	"
TRYPAN RED.				
1	10	68	Loc. and gen. tbc.	None
1	10	82	" "	"
1	10	68	" "	"
1	10	82	" "	"
1	6	35 (killed)	" "	"
1	10	75 "	" "	"
1	10	107	" "	"
62	2	72 (killed)	" "	"
62	2	72 "	" "	"
62	2	72 "	" "	"
CONTROLS FOR TRYPAN BLUE AND TRYPAN RED SERIES.				
		63	Loc. and gen. tbc.	
		31	" "	
		82	" "	
		68	" "	
		64	" "	

Within this area was an irregular zone showing almost no stain, but in which fat droplets and cells containing fat could be demonstrated by the use of fat dyes. Then the innermost portion of the tubercle was usually deeply stained, having either a homogeneous or a finely granular appearance. This inner part of tubercles, as well as the large necrotic areas in liver and spleen, had a different and brighter color than the normal portions of the organs. The giant cells, when present, showed a diffusely stained protoplasm and more deeply stained nuclei. Both in the giant cells and in the central portion of the tubercles, acidfast bacilli could be demonstrated by means of the Ziehl-Nielssen stain. In most of the guinea-pigs in this experiment the dye injections were begun on the day following the infection and were continued at intervals of four or five days for two months. In spite of the early beginning of treatment and the fact that the animals were kept saturated with the dyes for the first two months, all animals developed a local and general tuberculosis, except two which died early of an accidental infection. No influence on the tuberculous process, therefore, could be observed, unless we may regard as a favorable influence the fact that the treated animals lived somewhat longer than the controls. In a disease as irregular and freakish in all its manifestations as tuberculosis, I cannot regard length of life as an indication of curative influence, unless the autopsy findings indicate some definite tendency to healing or arrest of development of the process. In every case in this experiment the autopsy showed an active and advancing tuberculosis. One experiment was made to determine what would be the effect of one maximum dose of trypan blue very early in the disease. Four guinea-pigs were therefore inoculated subcutaneously with human tubercle bacilli and the next day each received an intraperitoneal injection of 10 c.c. of 1 per cent solution of trypan blue. Three of these pigs died during the first week or two from the toxic action of the dye, but the fourth lived 120 days, showing at autopsy a very pronounced local and general tuberculosis.

Through the kind courtesy of Dr. McCurdy I received for trial a bottle of the "vitalrothzenkerfixierbar," which he had used in some of his experiments. Six tuberculous guinea-pigs were treated with 1 per cent solution of this dye in the same way as with the trypan blue. The effect on the normal tissues was very similar to that of trypan blue and trypan red, certain connective tissue cells exhibiting deep red granules such as are not seen after staining fixed tissues. The tubercles, on the other hand, were much less deeply stained than the normal tissues and much less deeply than with either trypan blue or trypan red. No effect on the tuberculous process was observed.

We must therefore conclude that trypan blue, trypan red, and "vitalrothzenkerfixierbar" have no therapeutic effect on experimentally produced tuberculosis in guinea-pigs.

The distribution of these dyes is so interesting that one can scarcely help theorizing a little on its meaning. The granular stain in certain fixed connective tissue cells, which is the normal response to the stimulus of these dyes, has been the subject of much difference of opinion among workers with these dyes. Goldmann considered these granules as organula or vital granules of the cell, like the plasmosomes of Ehrlich, vitally stained by the dyes which flow in solution in the blood stream. Subsequent workers have gradually worked away from this hypothesis of Goldmann's; Kyes[1] suggests that the action may be phagocytic, since the cells which show the granular stain are the cells most actively engaged in phagocytosis; and McCurdy[2] states definitely that "the presence of granules of a lipoid-insoluble dye in any cell is an indication of phagocytic capacity in such a cell, the granules being formed by phagocytosis of the dyes from the colloidal solution in which they are present in the lymph." Evans,[3] also, in a report before the American Association of Anatomists in 1913 states that the taking up and storing of dye particles by certain cells is identical with the phagocytosis of larger particles by the cell. Phagocytosis seems the most reasonable explanation of the normal granular stain. In tubercles, in all injured, dead, and necrotic tissue on the other hand, we have a different type of staining—a diffuse, homogeneous, structureless stain, generally of a different and less intense color than that present in the granules. Goldmann regarded this as due to the solution of the granules. McCurdy speaks of it as a "passive type of staining in which nucleus and protoplasm are stained diffusely with no architectural details being shown beyond a darker stain in the nucleus." He states that every cell thus stained is permanently injured, and that the stain depends on the mutual affinity of the dye and albuminous degeneration products which may be formed before any morphological change has taken place that is demonstrable by postmortem methods. It has seemed to me possible that a portion of these diffusible colloidal dyes, on entrance into the animal body, change their character to that of a more coarsely colloidal state, the particles of which are readily taken up by phagocytosis, while the rest of the dye circulates in solution in the blood serum and in the lymph and readily permeates injured and dead tissues. It is well known that however thoroughly saturated the tissues become, the blood never contains more than a minimal quantity, merely sufficient to show a faintly blue ring at the periphery of a drop of blood placed on a sheet of white filter paper. The color of this ring is suggestively similar to that seen in injured and dead tissue.

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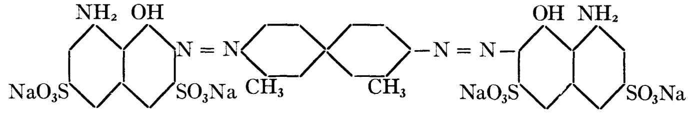
[1] Personal communication. Manuscript not yet published.

[2] By permission, from unpublished MS. To be published in Nissl-Alzheimer's *Hist. und histopath. Arbeiten über die Grosshirnrinde.*

[3] Abstract of report published in *Anat. Record,* 1914, 8, p. 98.

Since trypan blue and trypan red so effectively penetrate the tubercle and yet are so ineffective in treatment of the disease, it occurred to me that their bactericidal and therapeutic power might perhaps be increased by substituting for the sodium sulfonate radicals the sulfonate of other and more bactericidal metals.

For this purpose, silver, copper, mercury, and iron were selected, and Dr. Walter Fraenkel made the desired substitutions for me according to the following methods: The free dye acid was formed from trypan blue dye by covering 5 gm. of the salt with 100 c.c. of fuming HCl, stirring well and allowing it to stand overnight. After dilution with 25 c.c. of water, it was filtered; the precipitate was washed first with 25 per cent HCl and then with equal parts of alcohol and ether. After drying, the dye acid appears in the form of shiny bronze needles, which, unlike the salt, are soluble in methyl alcohol and slightly in acetone and in equal parts of alcohol and ether. The formula of trypan blue is:



Copper trypan blue.—Copper hydroxid ($Cu(OH)_2$) is freshly prepared by adding NaOH to a solution of $CuSO_4$, being careful not to precipitate all the copper. The precipitate is then washed several times with water and then mixed with 3 gm. of the free dye acid and evaporated over the water bath to complete dryness. The residue is extracted in a Soxhlet apparatus with methyl alcohol to remove any unused free dye acid, and the small amounts of $CuSo_4$ and $CuCl_2$ which may be present. After drying, the residue is dissolved in the cold by shaking with about 100 c.c. of water, and filtered, and the filtrate dried in Erlenmeyer flasks by passage of dried air at a temperature of 40°–60° C. The amount of copper present is 12.7 to 12.8 per cent and the formula is the same as that of trypan blue except that Cu takes the place of Na in each sulfonate group. It consists of bright bronze crystals, easily soluble in water.

Silver trypan blue.—Freshly precipitated silver oxid is carefully freed from NaOH and added to each of two 100 c.c. flasks containing 1.5 gm. of the free dye acid and about 60 c.c. of water. The two flasks are shaken for 5 hrs. in a shaking apparatus. The contents are then filtered and dried at a temperature not exceeding 50° C. The residue is extracted with methyl alcohol and then with acetone. The silver trypan blue thus obtained is a glistening bronze powder having 33.3 per cent silver. The formula is the same as that of trypan blue, except that silver takes the place of the sodium.

Iron trypan blue.—Three grams of free dye acid are mixed with iron oxid and 250 c.c. of water and heated with frequent stirring to dryness. The rest of the procedure is the same as used in making the copper salt and the result is a bright bronze powder containing 7.6–7.9 per cent of iron, and having iron in the trypan blue formula in place of the sodium.

Mercury trypan blue.—Two mercury salts of trypan blue were made, a mercurous and a mercuric. The mercurous salt was made by dissolving 3 gm. of trypan blue in 100 c.c. of water and adding an excess of mercurous nitrate. A finely divided bright blue precipitate results, which is very difficult to filter. The precipitate is washed on the filter with HNO_3 and then with water, and dried in the desiccator. The mercurous salt is insoluble in water and organic solvents and breaks down in alcohol, ether, and other organic compounds. When dried, it forms a bright blue powder containing 48 per cent of mercury. The mercuric salt of trypan blue is prepared in the same way as the mercurous salt except that mercuric nitrate is used instead of mercurous nitrate. The precipitate, after filtering and drying, is bronze colored and insoluble in water and all organic solvents. It can be made also by shaking mercuric oxid with the free dye acid for 3–4 hours. After filtering, it is washed with 1 per cent HNO_3 until excess of mercuric oxid is removed, then with water, and extracted in the Soxhlet apparatus with methyl alcohol and then with acetone. This salt contains 31.6–31.7 per cent mercury. Since both of the mercury salts are insoluble in water, it was necessary to use them in a fine suspension. This suspension was so fine that there was no appreciable settling out of the dye in weeks.

The bactericidal action of these salts of trypan blue was first tested by mixing 0.1 c.c. of a dilute filtered suspension of human tubercle bacilli with 5 c.c. of dye solution, and allowing the mixture to stand for 24 hrs. at incubator temperature. One drop of the mixed dye and bacterial suspension was then diluted with 5 c.c. of sterile normal salt solution and injected subcutaneously into guinea-pigs. The results are stated briefly in Table 2 (p. 505).

The two blanks in the mercury salts are due to the fact that by a mistake, these pigs received a later injection of tubercle bacilli which had been exposed to $CuCl_2$, and a local tubercle developed at the site of this later injection, followed by a generalized infection, causing the death of the animals. Since no tubercle developed at the site of the first injection, it may be assumed that the infection to which they succumbed was due to the second injection and that the mercury salts have considerable bactericidal or at least inhibitory power over the human tubercle bacilli. This, of course, might readily be expected, since we have here a 3 per 1,000 concentration of mercury in the mercuric salt and a 7 per 1,000 in the mercurous salt, while in a 1 per 1,000 solution of $HgCl_2$ the concentration of mercury is only about 0.7 per 1,000. None of the other salts of trypan blue used exhibited any marked bactericidal influence over the tubercle bacilli, altho the local tubercle developed rather slowly in some of the animals and in one of the pigs infected with the silver trypan blue treated bacilli, no local tubercle developed.

TABLE 2.

Dye Salt	Concentration Used	Time before Local Nodule	Subsequent Treatment	Time before Death	Autopsy Findings
	Per Cent	Days		Days	
Copper trypan blue...	1	18	Lithium carmine	115	Sev. loc. and gen. tbc.
	1	11	Brilliant cresyl blue	130	Sev. loc. and slight gen. tbc.
	1	11	Brilliant cresyl blue	112	Sev. loc. and gen. tbc.
Silver trypan blue....	1	18	Brilliant cresyl blue	121	Slight loc. and slight gen. tbc.
	1	46	Methylene blue	177	Sev. loc. and gen. tbc.
	1	None	Brilliant cresyl blue	121	No loc., sev. gen. tbc.
Iron trypan blue.....	1	40	None	156	Sev. loc. and gen. tbc.
	1	26	"	125	" " "
	1	40	"	109	" " "
	Hg				
Mercurous trypan blue	0.7	None	"	120	No loc., only pulmonary tbc.
	"	"	"	......	....
	"	"	"	240 (killed)	No tbc.; condition excellent
Mercuric trypan blue..	0.3	"	"	......	....
	"	"	"	264 (killed)	No tbc.; condition excellent
	"	"	"	240 (killed)	No tbc.; condition excellent
	Normal				
Controls NaCl.......	0.9	26	"	73	Loc. and gen. tbc.
	"	26	Pyrrhol blue	137	" "
	"	26	None	93	Slight loc., sev. gen. tbc.
	"	19	"	112	Sev. loc. and gen. tbc.
	"	19	"	118	Slight loc., sev. gen. tbc.

Experiments to determine the penetrating power of metallic salts of the dye showed that the silver and iron salts penetrated the tubercles as easily and more specifically than does the trypan blue itself, since the normal tissues were usually quite unstained by these salts while the tubercles and necrotic areas were nearly always deeply colored. The copper salt and the two mercury salts apparently do not penetrate the tubercles at all, nor indeed do they stain the normal tissues. This may be due to the fact that the high local toxicity of these salts made it necessary to use much smaller doses and much more careful administration than any of the other salts required. It may be, however, and more probably is, due to a local breaking down of these dyes into more or less insoluble and difficultly diffusible compounds, which remain at the point of injection. This may also explain the extensive necrosis and sloughing of the skin so common at the point of injection of these salts, and very rarely occurring with trypan blue or the silver and iron salts.

To determine whether these metallic salts of trypan blue would be of any value therapeutically in tuberculosis, a number of guinea-pigs were inoculated with a culture of human tubercle bacilli, and three days later, treatment was begun by subcutaneous injections of solutions of these salts.

Copper trypan blue.—On account of the necrotizing action of the copper, 0.5 and 1 c.c. of 0.1–0.25 per cent solutions of the dye were used.

1. A total of 42.5 mg. of dye or 5.44 mg. of copper was given in 28 injections. Pig died 77 days after inoculation. Local gland slightly enlarged but not caseous. Liver, spleen, and lungs filled with tubercles. Thick white exudate over pleura, pericardium, and peritoneum.

2. A total of 45 mg. of dye or 5.76 mg. of copper was given in 29 injections. Pig died 82 days after inoculation. Glands in axilla enlarged and caseous. Spleen, liver, and lungs filled with tubercles.

3. A total of 70.5 mg. of dye or 9 mg. of copper was given in 34 injections. Pig died 98 days after infection. Local gland slightly enlarged, not caseous. Liver, spleen, and lungs filled with tubercles.

4. A total of 46.5 mg. of dye or 5.95 mg. of copper was given in 29 injections. Pig died 103 days after infection. Local tubercle very small and not caseous. Spleen and liver large and necrotic. Lungs filled with tubercles.

5. Only 7 injections given and pig died of acute infection. No tuberculosis.

6. A total of 47.7 mg. of dye or 6.1 mg. of copper was given in 29 injections. Animal died 106 days after inoculation. No local tubercle. Spleen, liver, and lungs filled with tubercles.

Silver trypan blue.—The toxic dose of this salt having been determined by injections into mice, 0.5 c.c. of 0.1 per cent solution was decided upon as the maximum safe initial dose, this being later increased to 2 c.c. of a 0.5 per cent solution.

1. A total of 26 mg. of dye or 8.6 mg. of silver was given in 9 injections. Death occurred 23 days after inoculation. A local, slightly enlarged, but not caseous gland was the only indication of the development of the tuberculous infection.

2. A total of 103 mg. of dye or 34.3 mg. of silver was given in 18 injections. The pig died 44 days after infection, showing a local and general tuberculosis. The tubercles were all stained deep blue, while the normal tissue appeared unstained.

3. A total of 103.5 mg. of dye or 34.48 mg. of silver was given in 18 injections. Animal died 56 days after infection, with slight local and marked general tuberculosis. All tubercles were blue, normal tissue unstained.

4. A total of 138.5 mg. of the silver trypan blue or 46.1 mg. of silver was given in 21 injections. Death occurred 58 days after inoculation, autopsy showing slight local but very marked general tuberculosis. Tubercles were bright blue and all other tissues very red.

5. This pig received 22 injections of 140.5 mg. of dye, or 46.8 mg. of silver. Death occurred 58 days after infection, with slightly enlarged, non-caseous local glands, the spleen, liver, and lungs showing many definite and some caseous tubercles.

Iron trypan blue.—The initial dose, as in the silver trypan blue, was 0.5 c.c. of a 0.1 per cent solution, gradually increased to 2 c.c. of a 0.5 per cent solution.

1. A total of 149 mg. of dye or 11.77 mg. of iron was given in 23 injections. Death occurred 61 days after infection. Local glands were slightly enlarged, but non-caseous; spleen, liver, and lungs were filled with tubercles. All tubercles blue, the other tissues appearing unstained. No blue-stained bacilli seen.

2. This pig received 23 injections, totaling 149 mg. of dye or 11.77 mg. of iron. Death occurred 62 days after inoculation. Local glands were moderately enlarged and caseous; liver, spleen, and lungs markedly involved. All tubercles were deeply stained, even the caseous centers of tuberculous glands being blue.

3. A total of 189 mg. of the dye and 14.9 mg. of iron was given in 27 injections. The animal died 73 days after inoculation. A local gland was slightly enlarged and caseous, while liver, spleen, and lungs were filled with miliary tubercles and necrotic areas.

4. A total of 164 mg. of the dye or 12.95 mg. of iron was given in 28 injections. Death occurred 79 days after inoculation. Local glands slightly enlarged and hard, not caseous. Spleen large and necrotic. Liver but slightly involved. Lungs contained a few small tubercles. All tubercles and necrotic areas were blue.

5. A total of 209 mg. of the dye or 16.5 mg. of iron was given in 29 injections. The pig died 104 days after infection, showing a very small, hard, local tubercle. Spleen and liver were filled with tubercles and necrotic areas. The lungs were packed with tubercles, many containing cavities.

Mercury trypan blue.—In the therapeutic experiments, only the mercuric salt was used, the stock suspension being injected subcutaneously. The individual doses represented 0.2–0.8 mg. of Hg corresponding to 0.004–0.016 grain of $HgCl_2$ or 0.0035–0.014 grain of calomel.

1. A total of 10.56 mg. of dye or 3.33 mg. of Hg was given in 23 injections. Pig died 61 days after infection. Two slightly enlarged, but not caseous glands were found in groin. Spleen, liver, and lungs showed only a few small tubercles. The tuberculous process was not far enough advanced to be considered the cause of death. Neither tubercles nor normal tissues appeared stained with these dyes.

2. This pig received 26 injections, making 10.89 mg. of dye or 3.44 mg. of mercury, and died 71 days after infection. No local nodules. A few small tubercles in liver, spleen, and lungs. No dye seen anywhere.

3. This pig received 29 injections of 11.22 mg. of the dye and 3.54 mg. of mercury. Death occurred 87 days after infection. Autopsy showed slight enlargement of local glands; liver and spleen normal; lungs congested and presenting a few small tubercles. No dye seen.

4. Same amount and same number of injections as in No. 3. Death took place 117 days after inoculation. The local glands were slightly enlarged and the spleen and lungs well filled with tubercles.

5. In this animal, the dye injections were begun 20 instead of 3 days after infection. A total of 5.94 mg. of dye or 1.88 mg. of mercury was given in 23 injections. The pig died 142 days after inoculation. Autopsy showed slight local, but marked general tuberculosis.

It may be noted as curious that in all the animals treated with these salts of trypan blue, the local tuberculous process is either slight or absent, while the organic involvement is much more pronounced. It is difficult to say whether this marked variation from the ordinary course of development of tuberculosis in the guinea-pig is due to any action of the metals, possibly stimulating phagocytosis. As Table 3 shows, the animals treated with copper trypan blue and with mercuric trypan blue lived considerably longer than the controls and longer than the animals treated with the other salts. Of more importance seems the fact that the animals treated with the mercury salt, with the single exception of No. 5 in which the treatment was begun late, had a very slight tuberculous involvement, either local or general, and showed many symptoms suggesting chronic mercury poisoning.

TABLE 3.

SHOWING DURATION OF LIFE IN THESE EXPERIMENTS.

Controls	Copper Trypan Blue	Silver Trypan Blue	Iron Trypan Blue	Mercuric Trypan Blue
Days	Days	Days	Days	Days
82	77	23 (not tuberculosis)	61	61
71	82	44	62	71
97	98	56	73	87
....	103	58	79	107
....	106	58	104	142
Av.....76⅔	93⅕	47⅘	75⅘	97⅗

The silver and iron salts of trypan blue penetrate the tubercle as well as or better than does the sodium salt; the normal tissue is nearly unstained and under the microscope shows but few of the cells filled with blue granules which are so characteristic of the tissues of the trypan blue stained animals. The copper and mercury salts of the dye, on the other hand, have failed to penetrate the tubercle or to stain the normal tissues. Since the mercury salt is insoluble and was used in a fine suspension which settled out very slowly, it seemed not unlikely that this insolubility might be the reason for its slight diffusibility and for the fact that it has failed to stain any of the tissues. The copper salt, on the other hand, is readily soluble in water. At first it seemed possible that the necessarily minute doses might explain the lack of distribution. Later this explanation seemed insufficient and the thought occurred to me that the dye, on coming in contact with the body tissues, might flock out of solution, or at least become coarsely colloidal, in which state it might be less readily taken up by the cells and by the lymph. The marked necrotizing action of these salts was a factor strongly favoring the idea of retention at point of injection, and it was also found that egg albumin and pieces of fresh tissues added to a solution of the dye in a test tube caused a separation of the solution into two definite layers, a thick, heavy layer at the bottom and a clear layer at the top, which might be water-clear or somewhat stained. In order to discover what happened when the copper salt was injected subcutaneously, a lethal dose was injected into three pigs. One received 5 c.c., the second received 8 c.c., and the third had 10 c.c. of a 1 per cent solution. The guinea-pigs lived from 12 to 20 hrs. Within this time trypan blue would have disappeared from the point of injection and been distributed over the body. In these three pigs the organs were completely unstained and not even the skin beyond the natural spread of the fluid was stained, while a mass of soft gelatinous dark blue substance filled the entire groin and saturated the subcutaneous tissues in this region, but had not even penetrated to the underlying muscle. The failure of the copper dye to distribute itself seems, therefore, to be due not so much to the small doses as to the nature of the dye compound, which is such that it falls out of solution when it comes in contact with the protein of the tissues. A similar fact was noted by Corper[1] who observed that soluble copper salts became colloidal on coming in contact with living tissues.

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[1] Wells, DeWitt, and Corper. Unpublished manuscript, to appear in the *Ztschr. f. Chem.*

Some attempts have been made to determine whether these heavy metal salts of trypan blue were carried into the internal organs as such or even whether the metals were carried in at all. First the tissues from a pig treated with copper trypan blue and those from a pig treated with silver trypan blue were extracted by Dr. Corper in an effort to analyze the extracted dye for the metals. No copper, or not sufficient copper for determination, was found in the organs of the pig treated with copper; and in the silver trypan blue tissues the dye was found so firmly fixed in the cells and tissues that it could not be removed by any of the methods used. I have therefore tried certain biochemical methods for determining the presence of these metals. The fresh tissues of a guinea-pig treated with silver trypan blue were dropped into the pyrogallic acid-formalin mixture used in the Cajal and other methods for the reduction of silver in the tissues. Other tissues were fixed in Zenker's solution, which removes or fades the dye, and were then examined for silver granules in the tissues. By neither of these methods was I able to determine with any degree of certainty the presence of silver, either in the tuberculous or in the normal tissue. Sections of alcohol-fixed tissues from a pig treated with copper trypan blue were placed, some in potassium ferrocyanid, which should stain the copper-containing parts red, some in solution of ammonium hydrosulfide, in which the copper-containing parts should become yellowish brown, and some in dilute water solution of hematoxylin, which should stain the copper-containing parts deep blue. By none of these methods was I able to find a trace of copper. While the iron trypan blue was usually quickly absorbed and distributed over the body, in some of the animals long treated with this salt, especially if the solutions were not always fresh, a reddish deposit suggesting colloidal iron was found around the point of injection. Sections of tissues from guinea-pigs treated with iron trypan blue were exposed to the ammonium hydrosulfide and potassium ferrocyanid as suggested by Macallum, in which the iron-containing parts should appear green to black after the sulfide, but become blue after the ferrocyanid. Since all of these methods have been negative, it seems probable that these salts of trypan blue are easily broken down and that the metals are not carried by the dye to the internal organs. This fact may explain the failure to obtain any favorable therapeutic results from the use of these heavy metal salts of trypan blue.[1]

SUMMARY AND CONCLUSIONS.

Trypan blue and trypan red readily penetrate the tubercle in all stages of its development, thus showing that it is possible to penetrate the avascular tubercle by chemicals introduced either subcutaneously, intravenously, or intraperitoneally.

Trypan blue and trypan red do not penetrate the tubercle bacillus well, and do not kill it *in vitro* even after 24 hours' exposure to a 1 per cent solution.

In therapeutic doses, frequently repeated for long periods, trypan blue and trypan red seem to have no favorable or curative influence in experimental tuberculosis in guinea-pigs. In a single large, nearly lethal dose at the beginning of the infection they also have no favorable influence.

Silver trypan blue and iron trypan blue also penetrate the tubercle, but have no bactericidal and no therapeutic influence. It is doubtful whether the metals are carried in with the dye.

Copper trypan blue is soluble, but does not penetrate either the normal or the tuberculous tissues, and is probably changed to an insoluble form or a suspension colloid and retained at the point of injection.

Mercury trypan blue is insoluble, is strongly bactericidal in its action on the tubercle bacillus, but is too toxic for therapeutic use, since the pigs died apparently from chronic mercury poisoning rather than from the tuberculous infection, the tuberculous process being generally very slight. The findings with this salt, however, are suggestive, and further experiments with mercury salts will be made.

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[1] It gives me great pleasure to express my indebtedness to Dr. Walter Fraenkel for his assistance in making these metal salts and to Miss Sherman for the faithfulness and interest with which she has aided me in much of the tedious routine of this experiment.